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A cadherin-like protein influences Bacillus thuringiensis Cry1Ab toxicity in the oriental armyworm, Mythimna separata

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Abstract
Cadherins comprise a family of calcium-dependent cell adhesion proteins that act in cell–cell interactions. Cadherin-like proteins (CADs) in midguts of some insects act as receptors that bind some of the toxins produced by the *Bacillus thuringiensis* (Bt). We cloned a CAD gene associated with larval midguts prepared from *Mythimna separata*. The full-length cDNA (*MsCAD1*, GenBank Accession No. JF951432) is 5642 bp, with an open reading frame encoding a 1757 amino acid and characteristics typical of insect CADs. Expression of *MsCAD1* is predominantly in midgut tissue, with highest expression in the 3rd- to 6th-instars and lowest in newly hatched larvae. Knocking-down*MsCAD1* decreased Cry1Ab susceptibility, indicated by reduced developmental time, increased larval weight and reduced larval mortality. We expressed *MsCAD1* in *E. coli* and recovered the recombinant protein, rMsCAD1, which binds Cry1Ab toxin. Truncation analysis and binding experiments revealed that a contiguous 209-aa, located in CR11 and CR12, is the minimal Cry1Ab binding region. These results demonstrate that MsCAD1 is associated with Cry1Ab toxicity and is one of the Cry1Ab receptors in this insect. The significance of this work lies in identifying MsCAD1 as a Cry1Ab receptor, which helps understand the mechanism of Cry1Ab toxicity and of potential resistance to Bt in *M. separata*.

Keywords
toxins, CADs, recombinant protein

Disciplines
Agriculture | Entomology | Toxicology

Comments
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A cadherin-like protein influences *Bacillus thuringiensis* Cry1Ab toxicity in the oriental armyworm, *Mythimna separata*

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Summary

Cadherins comprise a family of calcium-dependent cell adhesion proteins that act in cell–cell interactions. Cadherin-like proteins (CADs) in midguts of some insects act as receptors that bind some of the toxins produced by the *Bacillus thuringiensis* (Bt). We cloned a CAD gene associated with larval midguts prepared from *Mythimna separata*. The full-length cDNA (*MsCAD1*, GenBank Accession No. JF951432) is 5642 bp, with an open reading frame encoding a 1757 amino acid and characteristics typical of insect CADs. Expression of *MsCAD1* is predominantly in midgut tissue, with highest expression in the 3rd- to 6th-instars and lowest in newly hatched larvae. Knocking-down *MsCAD1* decreased Cry1Ab susceptibility, indicated by reduced developmental time, increased larval weight and reduced larval mortality. We expressed *MsCAD1* in *E. coli* and recovered the recombinant protein, rMsCAD1, which binds Cry1Ab toxin. Truncation analysis and binding experiments revealed that a contiguous 209-aa, located in CR11 and CR12, is the minimal Cry1Ab binding region. These results demonstrate that MsCAD1 is associated with Cry1Ab toxicity and is one of the Cry1Ab receptors in this insect. The significance of this work lies in identifying MsCAD1 as a Cry1Ab receptor, which helps understand the mechanism of Cry1Ab toxicity and of potential resistance to Bt in *M. separata*.

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Introduction

Bacillus thuringiensis (Bt) Cry toxins exert their lethal actions by binding to receptors in insect midguts. So far, four midgut Cry protein receptors are known: cadherin-like proteins (CADs), aminopeptidase N (APN), alkaline phosphatase (ALP) and glycolipids (Griffitts and Aroian, 2005; Bravo *et al*., 2011). Two models outline the Cry toxin mode of action and associated mechanisms for resistance: the pore-formation and the signal transduction models. Both include similar initial steps for toxin solubilization, activation by midgut proteases, and binding to the primary receptor CAD. CAD genes have been cloned and sequenced from several insect species, including *Lepidoptera*, but not the oriental armyworm, *Mythimna separata* (Griffitts and Aroian, 2005).

The oriental armyworm is a polyphagous, migratory corn pest (Jiang *et al*., 2011), although not the main target of transgenic Bt corn expressing the Cry1Ab toxin in China. The use of Bt corn has rapidly increased (James, 2010), which exerts selection pressure towards the evolution of Cry resistance in insect larvae (Naranjo, 2010). Several species are resistant to Bt toxins, including *Plutella xylostella*, *Helicoverpa punctigera*, *Pectinophora gossypiella* and *Helicoverpa armigera* (Yang *et al*., 2009; Downes *et al*., 2010; Liu *et al*., 2010; Dhurua and Gujar, 2011). GM maize expressing Cry1Ab proteins effectively controls *M. separata*, but some individuals survive in the field, which may indicate resistance genes (Chang *et al*., 2007). *M. separata* has lower sensitivity to the Cry1Ac toxin than *H. armigera* (Yun *et al*., 2004), probably because of differences in Cry1Ac binding affinity to midgut receptors. Resistance to Cry toxins is closely related to changes in their receptors, and mutation of the CAD gene is a mechanism of resistance evolution (Han *et al*., 2009). Because this is a migratory pest species, with seasonal, long-distance, multi-generation round-trip migration in China (Jiang *et al*., 2011), we foresee the possibility that resistance to Cry toxins could emerge in *M. separata* and quickly become geographically wide spread throughout its migratory region. This unwelcome possibility increases

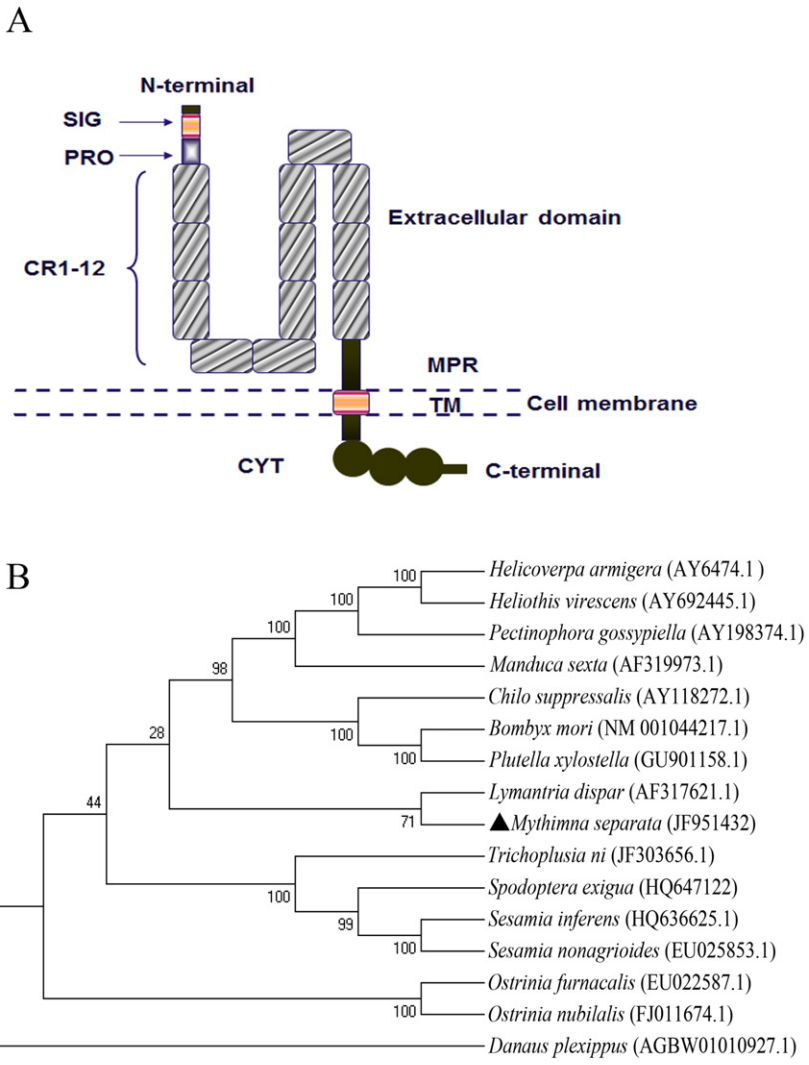

Fig. 1. A sketch of the MsCAD1 structure and identity analysis.
A. MsCAD1 signal peptide (SIG), proprotein region (PRO), cadherin repeats (CR1-12), membrane-proximal region (MPR) and transmembrane regions (TM). The dashed line represents a cell membrane. The extracellular domain is shown outside the membrane and CYT denotes the cytoplasm inside the cell membrane.
B. UPGMA phylogenetic tree generated from cadherin amino acid sequences of lepidopteran insect species; the sequence reported in this paper is marked by a solid triangle.

interest in the relations between Cry toxins and CAD. Our investigation begins with the hypothesis that Cry toxins exert their insecticidal and possible resistance mechanisms through interactions with CAD in *M. separata*. Here we report on outcomes of experiments designed to test our hypothesis.

Results

Cloning and characterization of MsCAD1

The complete nucleotide and deduced amino acid (aa) sequences of *MsCAD1* are presented in Fig. S1 (GenBank Accession No. JF951432). The sequence included a 5′-untranslated region of 234 bp, followed by an initiation codon (ATG). The cDNA contained a 5274 bp open reading frame (ORF), encoding an *in silico* 1757 aa protein with a calculated molecular weight of 196.8 kDa and isoelectric point of 4.5. The non-ORF at the 3′-end is 134 bp and contains a polyadenylation signal.

The inferred amino acid sequence includes a signal peptide (SIG), a proprotein region (PRO), 12 cadherin repeats (CR1-12), a membrane-proximal region (MPR), two transmembrane regions (TM), and a cytoplasmic region (CYT), typical structural characteristics of insect CADs (Fig. 1A). Transmembrane prediction showed the calculated MsCAD1 aa sequence possesses two transmembrane regions. One composed of 22 aa residues at the N-terminus, a signal sequence typical for eucaryotes, and the other made of 23 aa residues between aa 1606 and 1628, where cadherin anchors onto the midgut epithelial membrane. The MsCAD1 is closely related to lepidopteran CADs, with sequence identity ranging from 49.1% to 61.8% (Fig. 1B).

Age- and tissue-related MsCAD1 expression by real-time fluorescence quantitative PCR (qPCR)

MsCAD1 expression was significantly affected by developmental stage, highest in instars 3–6 and lowest in newly

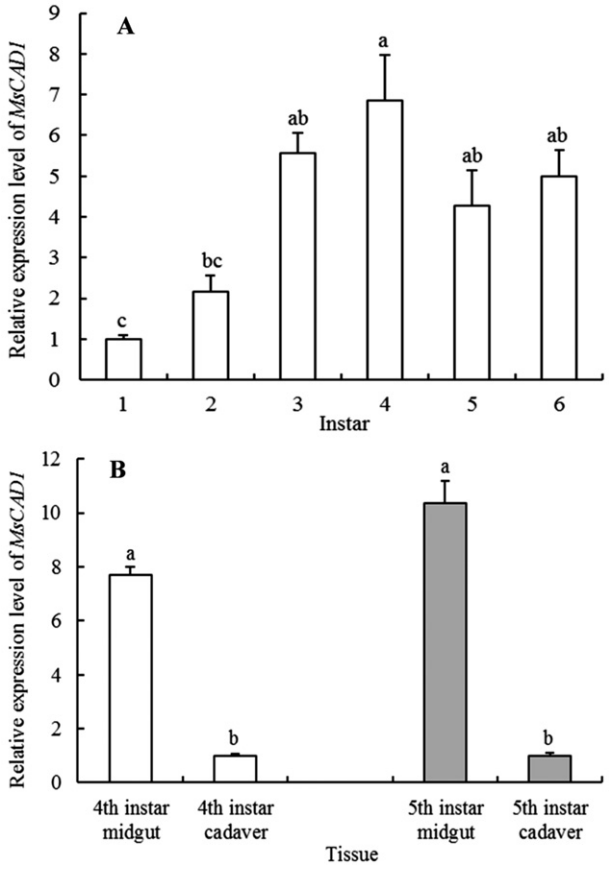

Fig. 2. *MsCAD1* expression profiles.
A. *MsCAD1* expression varies over instars.
B. *MsCAD1* expression is highest in midgut. Each histogram bar represents the mean ± SEM relative *MsCAD1* expression in the indicated instar (A) and tissue (B). Means sharing the same letters are not significantly different ($\alpha = 0.05$, Tukey's HSD).

hatched larvae (Fig. 2A). *MsCAD1* expression was much higher in midgut than in their larval cadavers (Fig. 2B).

MsCAD1 expression is reduced by dsRNA treatments

We determined the relative *MsCAD1* expression after larvae ingested, in separate groups, each of three dsRNA constructs, designated ds1, ds2 and ds3. Compared to control larvae, *MsCAD1* transcripts were substantially reduced in larvae 24 h after ingesting the high ds1 dose (Fig. 3A), and was reduced at 24 and 48 h after ingesting the high ds2 dose (Fig. 3B). Larvae fed on low ds1 and ds2 doses experienced smaller reductions in *MsCAD1* transcripts. *MsCAD1* transcripts were not affected in larvae fed on either high or low ds3 doses (Fig. 3C), similar to the control group fed on ddH_2O (Fig. 3D). There was no mortality among the experimental larvae.

MsCAD1 silencing influenced Cry1Ab toxicity

Experimental 3rd-instar larvae were placed on standard diets amended with 10 μl dsRNA (0.25 μg μl^{-1}) and controls were placed on standard diets amended with 10 μl water. The insects were allowed to feed for 18 h, after which they were fasted 2 h, then placed on standard diets amended with 45 μg Cry1Ab toxin/g diet. The duration of 3rd instars was not influenced by ds3 treatments, but was significantly reduced from about 3.7 days for controls to about 3.4 days (ds1) and 3.1 days (ds2; Fig. 4A). Larval mass significantly increased relative to controls in experimental larvae treated with low ds1 and ds2 doses (Fig. 4B), but again, not with ds3 treatments. Control larvae and larvae treated with ds3 experience about 60% mortality after exposure to dietary Cry1Ab, mortality decreased to about 55% in larvae treated with ds1 and to about 40% in larvae treated with ds2 (Fig. 4C).

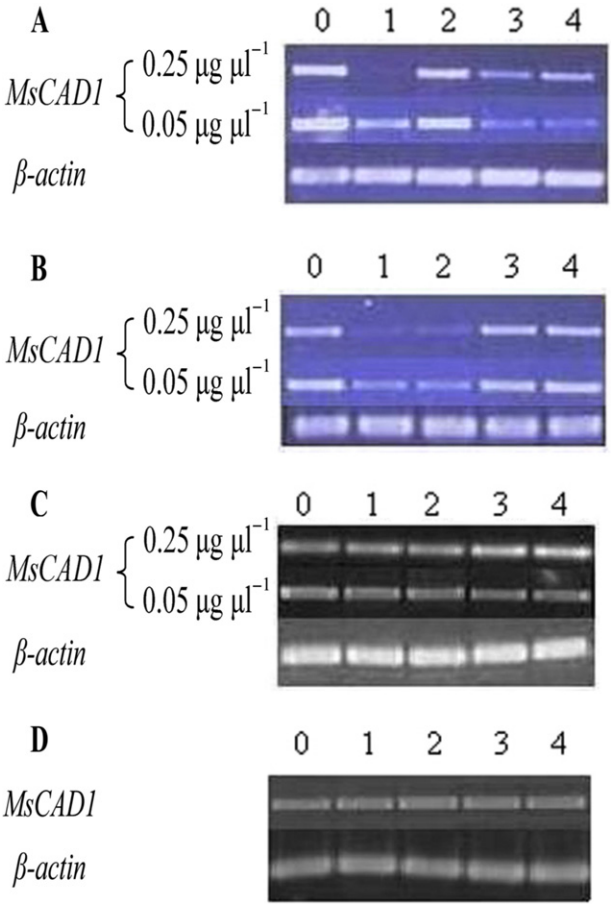

Fig. 3. Influence of dietary dsRNA on *MsCAD1* transcription. (A) dietary ds1; (B) ds2; (C) ds3; (D) ddH_2O. Total RNA was reversed transcribed to cDNA, and used as PCR templates, using *MsCAD1* or *β-actin* specific primers. The PCR products were separated on agarose gels and stained with ethidium bromide. Lane 0, positive control, larvae not fed on dsRNA. Lanes 1, 2, 3 and 4 represent results at 24, 48, 72 and 96 h post feeding respectively. *MsCAD1* expression was substantially reduced after ingesting high doses of ds1 or ds2, but not ds3 (lane A1, lanes B1, B2 and lanes C1–4). Dietary water did not influence expression (lanes D1–4).

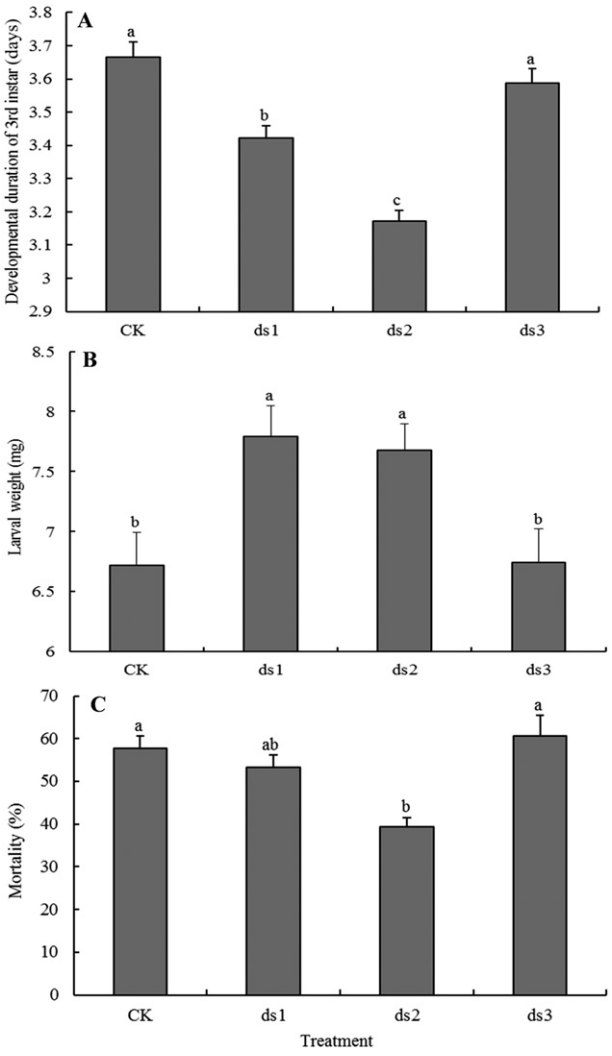

Fig. 4. Influence of ingested dsRNA followed by chronic dietary Cry1Ab on larval biological parameters.
A. Third instar duration. Histogram bars indicate mean days mean ± SEM.
B. Larval weight on the 7th day after treatment. Histogram bars indicate mean weight ± SEM.
C. Mortality on the 7th day after treatment. Histogram bars indicate mortality as proportions of total larvae in each treatment group ± SEM. Means with the same letter are not significantly different ($\alpha = 0.05$, Tukey's HSD).

Cry1Ab binds MsCAD1 in specific regions

The position of three truncated MsCAD1 peptides and their binding activity to Cry1Ab are presented in Fig. 5A. The SDS gel in Fig. 5B demonstrates the three peptides were purified. Truncated fragments 1 (CR9-12: 470 aa) and 3 (CR11-12: 209 aa) bound Cry1Ab (Fig. 5C, lanes 1 and 3), but fragment 2 (CR9-10: 274 aa) did not (Fig. 5C, lane 2).

Discussion

The results presented in this paper support our hypothesis that Cry toxins exert their toxicity through their interactions with CADs. Several points are germane. First, the nucleic acid and calculated aa sequences of the MsCAD1 is similar to other insect CADs. Second, we recorded substantial *MsCAD1* expression in midguts isolated from 4th- and 5th-instar larvae, and very low expression in their cadavers. Third, *MsCAD1* expression was reduced in dsRNA-treated larvae. Fourth, three life history parameters were substantially altered in dsRNA-treated larvae. Finally, Cry toxin bound to specific segments of the MsCAD1 protein. Taken together, these points indicate that Cry toxins influence *M. separata* through interactions with MsCAD1 protein.

Insect resistance raises concerns about sustainability of Bt biotechnology. Previous studies showed that Cry1A resistance in several lepidopteran species was associated with mutations of CAD genes, such as deletions in *Ostrinia nubilalis* (Bel *et al*., 2009), premature stop codons in *P. gossypiella* (Morin *et al*., 2003), and insertion and single aa mutations in *H. virescens* (Xie *et al*., 2005). Disruptions of lepidopteran CAD genes by transposable elements occur frequently, conferring Cry1Ac resistance (Fabrick *et al*., 2011). Yang and colleagues (2011) reported that altered expression of a midgut CAD was associated with Cry1Ab resistance. The situation is complicated, however, because other studies report that APNs are involved in Bt resistance in some insect species (Bravo *et al*., 2004). We infer that research on a wider range of species is needed to understand and potentially manipulate Cry resistance mechanisms in pest insects.

The arrangement of *M. separata MsCAD1* functional motifs, including SIG, CR, TM and CYT, is typical of insect CADs. Given their structural similarities (Bel and Escriche, 2006), it is likely that lepidopteran Bt-related CAD genes share evolutionarily conserved functions. In our view, the MsCAD1 fits into the general lepidopteran pattern.

As with other insects, *MsCAD1* is expressed predominantly in midgut tissue, but expression differs substantially among larval instars, reaching a peak in the 4th- through 6th-instars, similar to the expression of CAD genes in *P. xylostella* (Yang *et al*., 2012) and *Spodoptera exigua* (Lu *et al*., 2012). In terms of field application, this coincides with the finding that *M. separata* is most susceptible to Cry1Ab toxins during the 4th-instar (Wang *et al*., 2005). The high *MsCAD1* expression in 4th- to 6th-instars probably relates to an unrelated aspect of midgut physiology, but it may be one of the reasons why 4th-instar larvae are more susceptible to Cry1Ab toxins.

Experimental larvae treated with ds1 and ds2 experienced decreased susceptibility to the Cry1Ab toxin. The decreased susceptibility is due to reduced *MsCAD1*

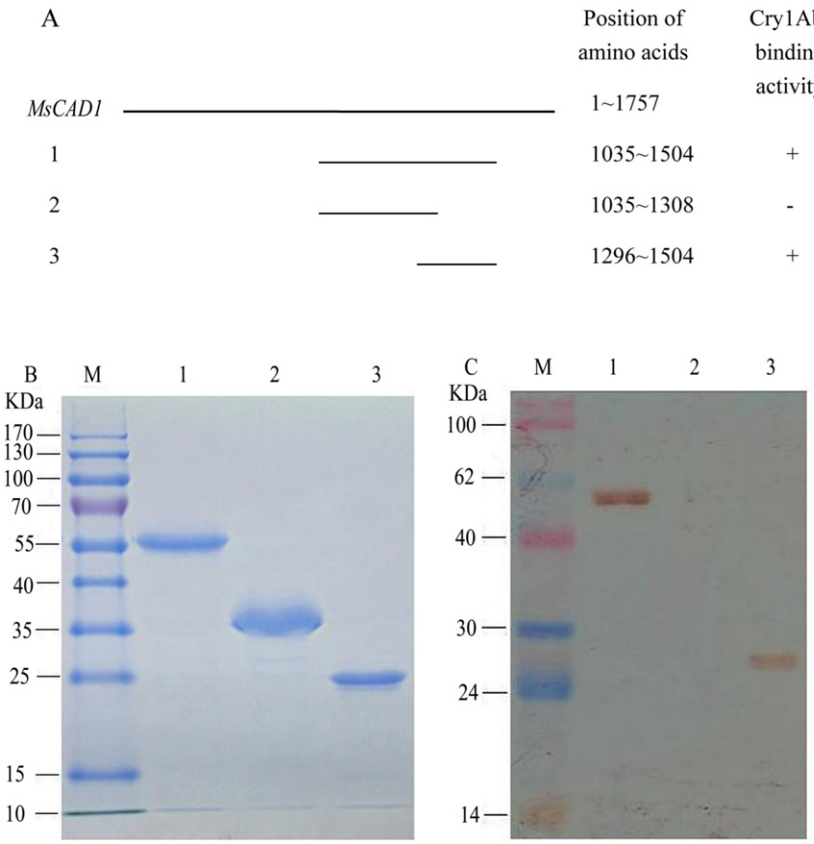

Fig. 5. Cry1Ab specifically binds two purified recombinant MsCAD1 fragments.
A. The positions of three truncated MsCAD1 peptides and their binding activities.
B. A SDS-PAGE gel showing each of the recombinant truncated peptides were purified.
C. A representative Western blot showing Cry1Ab specifically bound to fragments 1 and 3, but not 2.

expression. We generated three dsRNA constructs, two of which (ds1 and ds2) silenced *MsCAD1*. Neither *MsCAD1* transcript levels nor susceptibility to Cry1Ab toxin were affected by ds3. We infer the decreased *MsCAD1* expression was due to alignment of dsRNA fragments with *MsCAD1* transcripts, not due to adventitious events within larvae. Our view is supported by the results of our Western blot experiments, showing that MsCAD1 binds Cry1Ab within a binding region of 209 aa between aa residues 1296 and 1504, located in CR11-12. This is similar to other CADs, from which we suggest the 1–2 cadherin repeats near the MPR is the critical Cry1A binding region of lepidopteran CADs (Fabrick and Tabashnik, 2007).

Experimental procedures

Summary

All experimental work, including insect rearing, cloning a full-length *MsCAD1* cDNA, sequence alignment and analyses, qPCR, RNA interference, expression and purification of recombinant MsCAD1 fragments, insect bioassays and Western blot analyses, was accomplished using standard protocols detailed in Supporting Text 1.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Nucleotide sequence and deduced amino acid sequence of MsCAD1. The first nucleotide of the initiation codon is marked as '+1'. The initiation and stop codons are indicated by bold. Arrows indicate starting points of deduced structure domains. SIG, signal peptide; PRO, proprotein region; CR, cadherin repeat; MPR, membrane-proximal region; TM is transmembrane region; CYT, cytoplasmic region.
Fig. S2. Relative primer positions within the *MsCAD*1 transcript.
Table S1. Primer sequences used in cDNA cloning, real-time quantitative PCR, RNAi and the expression of recombinant MsCAD1.
Supporting Text 1. Detailed experimental procedures.